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Interfering with Hedgehog Pathway: New Avenues for Targeted Therapy in Rhabdomyosarcoma

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Abstract

Rhabdomyosarcoma (RMS) is the most frequent pediatric soft-tissue tumor accounting for about 7% of childhood malignancies. Multimodal therapy is the standard treatment for individuals with RMS but generally fails to cure high-risk group patients and can result in long-term side effects. Therefore, understanding the mechanisms driving RMS might help to find new candidate targets for more specific and effective therapeutic modalities. One of the molecular machineries, which is often deregulated in cancer and specifically involved in the tumorigenesis of RMS, is Hedgehog (Hh) signaling. There is increasing evidence that targeting this developmental pathway may hold promise in future treatment strategies for RMS. In this review, we discuss the contribution of the Hh pathway in RMS, the challenges of inhibiting this embryonic signaling in children with an update on recent preclinical data and ongoing clinical trials.

Introduction

A pioneering study of Nusslein-Volhard and Wieschaus in 1980 identified a locus which when mutated caused duplications of the anterior denticle band of drosophila body segments, resembling Hedgehog spines [1]. This work represents one of the milestones for developmental biology, which inaugurated decades of studies directed at the characterization of a new evolutionary conserved signaling pathway named Hedgehog (Hh) [2-6]. Indeed, Hh pathway plays pivotal roles in early embryonic pattern formation as well as in adult pattern maintenance, given its importance in cell fate specification, survival and proliferation of different cellular contexts [7, 8]. Therefore, it is not surprising to find deregulation of Hh signaling implicated in different cancer entities such as childhood tumors [9]. This might explain why Hh pathway activation is drastically attenuated in adult tissues where it mostly controls homeostasis of stem cell populations. Hence, specific Hh inhibition might be an attractive anti-cancer therapy in adulthood but raises the question on its applicability in pediatric malignancies such as rhabdomyosarcoma (RMS).

RMS accounts for the majority of soft tissue sarcomas in children and rarely occurs in adults, suggesting that failure of proper developmental processes might be at least in part responsible for development of this tumor. It is thought to arise from cells of the myogenic lineage, given the expression of early skeletal muscle differentiation markers, but the precise cell of origin is still under discussion. The two main subtypes of RMS are named alveolar (ARMS) and embryonal (ERMS) rhabdomyosarcoma, respectively. They show differences in histology, clinical outcome, localization, incidence and molecular characteristics [10]. The main genetic alteration of ARMS is the presence of chromosomal translocations leading to the formation of mainly two fusion proteins named PAX3/FOXO1 and PAX7/FOXO1 (in 80% of cases) [11]. In contrast, ERMS displays frequent mutations in the RAS pathway or deletions of tumor suppressor genes with a generally more heterogeneous genetic landscape [12]. Multimodal therapy including surgery, radiation and chemotherapy is the standard treatment for RMS. Despite improvements in the survival rate of patients with localized disease, the outcome for high-risk groups remains poor [13]. Therefore, there is a need to find new targets through a better understanding of the molecular mechanisms underlying this disease [14]. Here, we review the role of Hh signaling in RMS. We first describe the molecular components of the pathway and then provide evidence of its deregulation in this childhood cancer. We also summarize currently available mouse models of RMS involving genetic manipulation of Hh pathway. Finally, we focus on the clinical implications of targeting Hh signaling in RMS with an update of recent discoveries and new clinical trial approaches.

Molecular mechanisms of mammalian Hedgehog signaling

As most of the molecular circuits, the mammalian Hh signaling can be ‘dissected’ into its main components including ligands (Desert Hedgehog (DHH), Indian Hedgehog (IHH) and Sonic hedgehog (SHH)), an

inhibitory transmembrane receptor (Patched (PTCH)), a ligand-activated co-receptor (Smoothened (SMO)) and the down-stream effectors (Glioma-associated oncogene (GLI) transcription factors GLI1, GLI2 and GLI3)). Even though the exact role of the three Hh ligands in vertebrate is not fully understood, they can have overlapping roles or elicit different responses mainly depending on their spatial and temporal localization. For example, SHH is essential for limb development as well as neural tube formation [15, 16], IHH promotes chondrocyte proliferation and bone specification [17, 18], while DHH is mainly expressed in testis, where it is involved in male sexual differentiation [19]. A common feature of Hh ligands is their lipophilicity due to binding of a cholesterol molecule to the C-terminus and a palmitoyl group to the N-terminus. In particular, the cholesterol moiety is required for the autocleavage of the inactive precursor in an active N-terminal form before being released by Dispatched (DISP), a transmembrane transporter acting on the signal-sending cell [20, 21]. Binding of Hh ligands to the 12-transmembrane-span protein PTCH promotes ligand-receptor internalization and subsequent lysosomal degradation [22]. Consequently, SMO, a member of the G-protein-coupled receptor (GPCR) family, translocates to the mammalian primary cilium and releases the downstream GLI zing-finger transcription factors from suppressed of fused (SUFU) inhibition. In vertebrates, GLI1, GLI2 and GLI3, mediate the expression of Hh target genes, most of which are still unknown [23, 24]. GLI2 and GLI3 are the main Hh-regulated activator and repressor, respectively. They both contain a N-terminal repressive and C-terminal activating domain. The Hh Off-state allows the C-terminally truncated GLI3 to block the transcription of the Hh responsive genes. In contrast, Hh ligand-mediated activation of the pathway leads to processing of GLI full-length proteins in C-terminal transcriptional activators and degradation of the N-terminal repressor domain. Moreover, the balance between active and repressive proteins is tightly controlled by post-translational modifications and may be altered in cancer [25]. On the other hand, GLI1 is a constitutive activator lacking the N-terminal repressor domain and its expression is directly regulated by Gli2 in response to Hh ligands as well as by non-canonical Hh signaling [23, 26].

The 'self-control' of Hedgehog pathway

Several mechanisms modulate the response to Hh signals at different cellular levels. First, cell adhesion molecule down-regulated by oncogenes (CDO) and brother of CDO (BOC), are two transmembrane proteins, which act as 'helpers' of PTCH to bind the ligands and trigger Hh pathway activation [27]. A recent report has identified BOC as mediator of Hh-induced DNA damage stimulating medulloblastoma progression, indicating the importance of these proteins in controlling Hh signaling [28]. Growth arrest-specific gene 1 (GAS1) and Hedgehog-interacting protein (HHIP) are two vertebrate-specific cell surface proteins which positively and negatively regulate Hh distribution, respectively [25, 29, 30]. Second, different kinases, phosphatases and ubiquitin ligases can post-translationally modify Hh pathway members to control signaling activity. For instance, in absence of Hh ligands, GLI transcription factors are sequentially phosphorylated by protein kinase A (PKA), glycogen synthase kinase 3 β (GSK3-β) and

different members of casein kinase family (CKI). This allows their ubiquitination and subsequent proteasomal degradation of the C-terminal transactivation domains. This process is reverted by SMO activation, which limits GLI phosphorylation and leads to stabilization of the full-length or C-terminal activator forms [6]. Moreover, GLI1 and GLI2 can undergo acetylation acting as repressive signal whereas the histone deacetylase 1 (HDAC1)-dependent deacetylation does the opposite [31]. Finally, a further control of the pathway is offered by GLI-mediated transcription of three Hh components PTCH1, HHIP and GLI1, activating both positive and negative feedback loops. In this respect, their expression is considered as the most reliable readout of Hh pathway activation.

Role of Hedgehog pathway in RMS

The first link between Hh and cancer comes from studies of Gorlin Syndrome (also called Basal Cell Nevus Syndrome, BCNS), a heritable condition characterized by several developmental abnormalities and association with high risk to develop tumors, mostly multiple basal cell carcinomas (BCC), medulloblastoma, and RMS [32, 33]. Since its discovery in 1960, different researches attempted to identify the locus associated with this autosomal dominant disease. Finally, more than thirty years later, PTCH has been reported as the candidate gene responsible for BCNS and therefore as a tumor suppressor gene [34-37]. Subsequently, the generation of mice heterozygous for Ptch confirmed the involvement of Hh pathway over-activation in RMS tumorigenesis [38]. Accordingly, up-regulation of Hh target genes such as GLI1 and PTCH1 has been demonstrated by retrospective analysis of RMS patient samples or in human RMS cell lines [39-43]. Additionally, activation of Hh pathway seems to be specific for fusion negative RMS (NRMS) and significantly identifies patients with poor prognosis [40, 41, 44, 45]. Despite this recognized role of Hh in RMS, the contribution of the ligand-based signaling versus non-canonical pathway activation in sporadic RMS is still unclear. Discordant studies searching for inactivating mutations in PTCH or SUFU genes and amplifications in SMO or GLI loci have been published. For instance, a cytogenetic approach of 12 separate RMS patients identified 4 cases with losses in the chromosomal region containing the PTCH1 gene [46]. Similarly, a linkage analysis led to comparable conclusions in one third of NRMS analyzed [39]. Controversially, Calzada et al., did not detect mutations in the coding sequence of PTCH1 in 14 RMS sequenced [47]. This is corroborated by another study showing absence of PTCH1 loss-of-function mutations or SMO amplifications in 26 NRMS examined [41]. Also, a recent whole-genome sequencing (WGS) analysis on 16 RMS tumors ruled out the presence of mutations in components of the Hh pathway [48]. SHH immunoreactivity is not common in RMS and the higher Hh activity in NRMS compared to fusion positive RMS (PRMS) does not correlate with SHH mRNA levels, which are unchanged between the two subtypes [39-41]. However, we previously found a positive correlation of IHH and DHH mRNA levels with the expression of Hh target genes in RMS supporting the hypothesis of a ligand-mediated activation of Hh pathway [45]. Therefore, underestimation of the role of the other two Hh ligands might have led to misleading conclusions and further studies need to more carefully elucidate their role in RMS.

In summary, hyperactivity of Hh pathway is a common feature in the NRMS and only a subset of RMS exhibits mutations in components of the Hh pathway, which would potentially argue for non-canonical pathway activation. However, the role of other tumor-associated pathways, which can interplay with diverse components of Hh signaling and thereby contribute to its activation, is still poorly investigated.

Mouse models of Hedgehog-driven RMS

Investigation of the 'cell of origin' is one of the most intriguing research topics in RMS. To this end, gene-targeting tools developed over the last years have been used to generate animal models of RMS, highlighting the central role of Hh in tumorigenesis of this pediatric soft tissue sarcoma. In 1998 Hahn et al., established the first mouse model of RMS directly involving Hh pathway. The authors showed that Ptch1 haplodeficient mice develop RMS tumors with molecular features of the NRMS subtype although with low frequency [38, 49]. Accordingly, all RMS from $Ptch^{+/-}$ mice over-expressed Gli1 and Insulin-like growth factor 2 (Igf2). Furthermore, the epistatic function of Igf2 to Ptch1 was later confirmed in mice double mutant for Ptch1 and Igf2 ($Ptch^{+/-}$ and $Igf2^{+/-}$) [50]. In contrast to $Ptch1^{+/-}$ mouse models, ubiquitous activation of a mutated form of a Smo allele (Rosa26-SmoM2) leads to the generation of RMS with higher penetrance [51]. In addition, mice heterozygous for Sufu ($Sufu^{+/-}$) in $P53^{-/-}$ or $Ptch1^{+/-}$ background harbor RMS tumors to the same extend as $Ptch1^{+/-}$ mice [52, 53]. By contrast, $Sufu^{+/-}$ mice develop only microscopic skin lesions. This suggests that P53 knockout affects the tumorigenesis of Sufu $^{+/-}$ mice and that there is no genetic interaction between Ptch1 and Sufu loci in RMS tumorigenesis. Recently, Rajurkar et al., provided additional insights into the link between aberrant Hh pathway activation and the cellular context responsible for RMS tumorigenesis [54]. They reported that specific expression of SmoM2 in postnatal (P10) Shh-producing cells as well as in Gli1-expressing cells did not lead to RMS formation within 4 months. This was also true for forced expression of SmoM2, Gli2 (Gli2ΔN) alone or in combination with Gli1 in postnatal satellite cells (Pax7 positive). Therefore, the authors ruled out the possibility of Hh-induced postnatal RMS formation in Hh-expressing compartments and myogenic cells, which is consistent with another study suggesting the adipocyte lineage as the NRMS-initiating population [55]. Surprisingly, restriction of Smo-M2 expression to adipocytes resulted in 80% incidence of NRMS which is far higher than $Ptch1^{+/-}$ and $Sufu^{+/-}$;$P53^{-/-}$ mice. More important, RMS was the only type of tumor detected, providing a tool to investigate therapeutic strategies specifically for this tumor. Such a model might explain why RMS develops also in regions of the body lacking skeletal muscle (i.e. genitourinary and biliary tract) [55]. Controversially, Rubin at al. demonstrated that concomitant inactivation of Ptch1 and P53 ($Ptch1^{+/-}$,$P53^{-/-}$) in a wide range of cells of the myogenic lineage (satellite cells, early and more differentiated myoblasts) contribute to RMS, which is not the case of P53 loss alone [56]. In general, discrepancies in the tumor incidence for $Ptch1^{+/-}$ and SMO-M2 mice might reflect differences in signaling activity or suggest that they do not completely lie on the same axis to promote RMS tumorigenesis. Alternatively, the stage at which the pathway is switched on in these mouse models might account for

distinct susceptibility to tumor development. For example, the large population of uncommitted precursors present at the early embryonic phase might be an important source of RMS onset in Ptch1$^{+/-}$ mice during development [49]. All together, these findings underscore the crucial function of Hh pathway in RMS tumorigenesis even though the cellular context and the time at which the uncontrolled activation of the pathway becomes oncogenic is still not clear. Finally, different mouse models have been proposed as preclinical platforms for RMS-specific therapies (Figure 1). However, if these models truly recapitulate the human situation needs further clarification.

Targeting Hedgehog in RMS

Since the discovery of Hh more than 30 years ago, inhibition of its activity has emerged as a promising approach for cancer therapy. This is extremely relevant for tumors where aberrant activation of Hh signaling takes place including BCC, medulloblastoma and RMS. Interestingly, a high number of inhibitors targeting different components of the Hh molecular machinery are available to date (Figure 2). This includes ligand inhibitors (i.e, robotnikinin, 5E1 and MEDI-5304 neutralizing antibodies), Hh acyltransferase antagonists (i.e., RU-SKI 43), compounds targeting SMO (i.e., cyclopamine, Cur-61414, SANT1-4, LDE 225, GDC-0449, HPI 2-3, IPI 926, BMS-833923, ALLO1-2, Itraconazole), ciliogenesis inhibitors (CA1, CA2 and HPI-4) and GLI antagonists (i.e., GANT58, GANT61, HPI-1, forskolin and Arsenic Trioxide (ATO), glabrescione B) [57-59]. Notably, cyclopamine, a natural occurring compound targeting SMO, has been shown to be effective in reducing proliferation of RMS primary cells isolated from Ptch$^{-/+}$ mice or human RMS cell lines *in vitro* [60-62]. However, this effect was not recapitulated in tumor-bearing Ptch$^{-/+}$ mice, raising questions regarding its stability *in vivo* given its poor solubility in water and chemical instability [63, 64]. Therefore, the generation of new cyclopamine derivates or inhibitors of other components of Hh signaling opened new avenues for targeting this pathway. For instance, GANT-61, an inhibitor of GLI activity has been reported to reduce RMS tumor growth in the chick chorioallantoic membrane (CAM) assay and in xenograft mouse models, even though at high concentrations [62, 65, 66]. Similar effects have been observed for forskolin without significant side effects [67]. Also, betullic acid, a pro-apoptotic drug, has been proposed as a modulator of Hh signaling activity in RMS cell lines although this effect was cell type-dependent [68]. Nevertheless, the antitumorigenic effect of these two naturally occurring compounds is not specifically related to suppression of Hh pathway. Interestingly, primary cilium, which is indispensable for transducing Hh signaling in mammalian cells, is abnormally assembled in a subset of RMS [69]. This may account for Hh over-activation and therefore it represents an attractive drug target. Importantly, one of the biological limitations of RMS-directed therapies is the possible existence of functionally defined sub-populations of cells within the tumor having increased tumorigenicity, self-renewal and chemoresistance [70]. This is prominent for NRMS where the hierarchical model may be applied and has to be taken into account when developing novel treatment strategies [45, 71-74]. Indeed, we found that Hh inhibition might provide a promising anti-cancer stem cell (CSC) therapy in

NRMS, and therefore multistrategy approaches with bulk-reducing drugs may be more effective in tumor eradication, particularly for high-risk group patients [45]. Accordingly, LDE-225 alone and Vismodegib (Curis/Roche) in combination with a Notch inhibitor (R04929097) have entered a clinical trial for recurrent RMS and adult advanced RMS, respectively [75, 76]. This highlights the importance of studying developmental pathways in pediatric tumors, which might offer new options for future targeted therapies.

Challenges of targeting Hedgehog pathway and future directions

Currently, clinical trial strategies involving Hh pathway inhibitors include only SMO antagonists, based on the success in preclinical models for different cancer types. In particular, vismodegib has been recently approved by US Food and Drug Administration (FDA) for the treatment of locally advanced and/or metastatic BCC [77-79]. However, a case study of a 26-year-old man with metastatic medulloblastoma has shown only a transient response to GDC-0449 and the patient died 5 months later [80]. Further analysis of the biopsy post-treatment revealed the presence of a missense mutation in SMO (D473H) which was responsible for lowering the binding affinity to the drug [81]. Thereafter, additional SMO mutations as well as GLI2 and cyclin D1 (an Hh target gene) amplifications were found in mouse models of medulloblastoma resistant to SMO inhibitors [82, 83]. Surprisingly, over-activation of other oncogenic pathways such as IGF-1R-PI3K signaling has been proposed as a mechanism of resistance to SMO inhibition independently of genetic aberrations of Hh players [82]. This is particularly remarkable for RMS where the IGF-1R-PI3K pathway is widely over-expressed and might account for refractory response to Hh inhibition [84]. Indeed, rapamycin, an mTOR inhibitor, has been reported to prevent RMS tumor growth by inhibiting both PI3K/AKT/mTOR and Hh signaling [85]. More important, the inhibitory effect of GANT61 on RMS cell proliferation is strongly enhanced by mTOR antagonists or chemotherapeutic agents, suggesting that combination strategies involving Hh inhibitors might be a beneficial therapeutic modality [66]. However, different scenarios of non-canonical activation of Gli transcription factors may occur and account for SMO-independent activation of Hh signaling. For instance, bromo and extra C-terminal (BET) bromodomain proteins have been shown to regulate the GLI1-mediated transcription. Consequently, inhibition of BRD4 by JQ1 was able to impair tumor growth of a wide array of tumors resistant to SMO inhibitors such as BCC, medulloblastoma and atypical teratoid rhabdoid tumors (ATRTs) [86]. Accordingly, SNF5, another important chromatin remodeling protein that is often inactivated in human malignant rhabdoid tumors (MRTs), interacts with GLI1 and represses its activity [87]. Moreover, atypical proteinase kinase C (aPKC) phosphorylates GLI1 to increase its binding to the DNA [88]. Targeting this kinase in SMO resistant BCC tumors resulted in effective tumor eradication. Although such approaches might hold promise for many cancer types resistant to conventional SMO inhibitors, their therapeutic window remains incompletely addressed in childhood cancers. This is an important caveat given the indispensable function of Hh signaling during development. Therefore, it is not unexpected to find that short-term blocking of Hh pathway in young mice resulted in severe and irreversible side effects, specifically in the bones [89]. Also,

knockout for Ptch1 and Sufu as well as Gli1/Gli2 double mutants (lacking the DNA binding domain of GLI1 and GLI2) are not compatible with life in mice due to defects during neurogenesis and heart or lung abnormalities [38, 52, 90]. In contrast, mice homozygous for a Gli1 mutant allele have a normal phenotype suggesting that Hh inhibitors targeting specifically Gli1 might be well tolerated in children [90]. However, direct and specific targeting of transcription factors remains a difficult task and indeed the mechanism of action of the available GLI1 antagonists is not completely unraveled.

Conclusions

In summary, we have described the importance of Hh signaling in RMS, the most common soft tissue sarcoma in childhood. Consistent with a key role of Hh pathway during embryogenesis, its deregulation is commonly associated with RMS, mainly with NRMS. As a consequence, LDE-225 is currently in phase 1/2 clinical trial for progressive RMS albeit as single agent. This is in contrast to preclinical studies showing that combinatorial strategies might evoke better and more durable tumor responses. Although current clinical studies include only SMO inhibitors, investigations of other Hh-driven tumors have demonstrated that resistance to SMO inhibitors is frequently observed because of the presence of point mutations in SMO or amplifications of GLIs as well as compensatory mechanisms involving interplayed signaling acting downstream of SMO [91]. Probably, the broad-range of characterized SMO antagonists is linked to the presence of this protein on the cell surface, which made it easily identifiable through drug screenings [59]. Therefore, specifically targeting GLI transcription factors or their positive regulators might be a valid alternative, albeit challenging. However, one of the more directly applicable result from the studies of Hh signaling in RMS is the possibility for a better stratification of patients for a more personalized therapy. This includes selection of those that would benefit of Hh-directed therapy and might contribute to both restrict toxicities and boost RMS cure.

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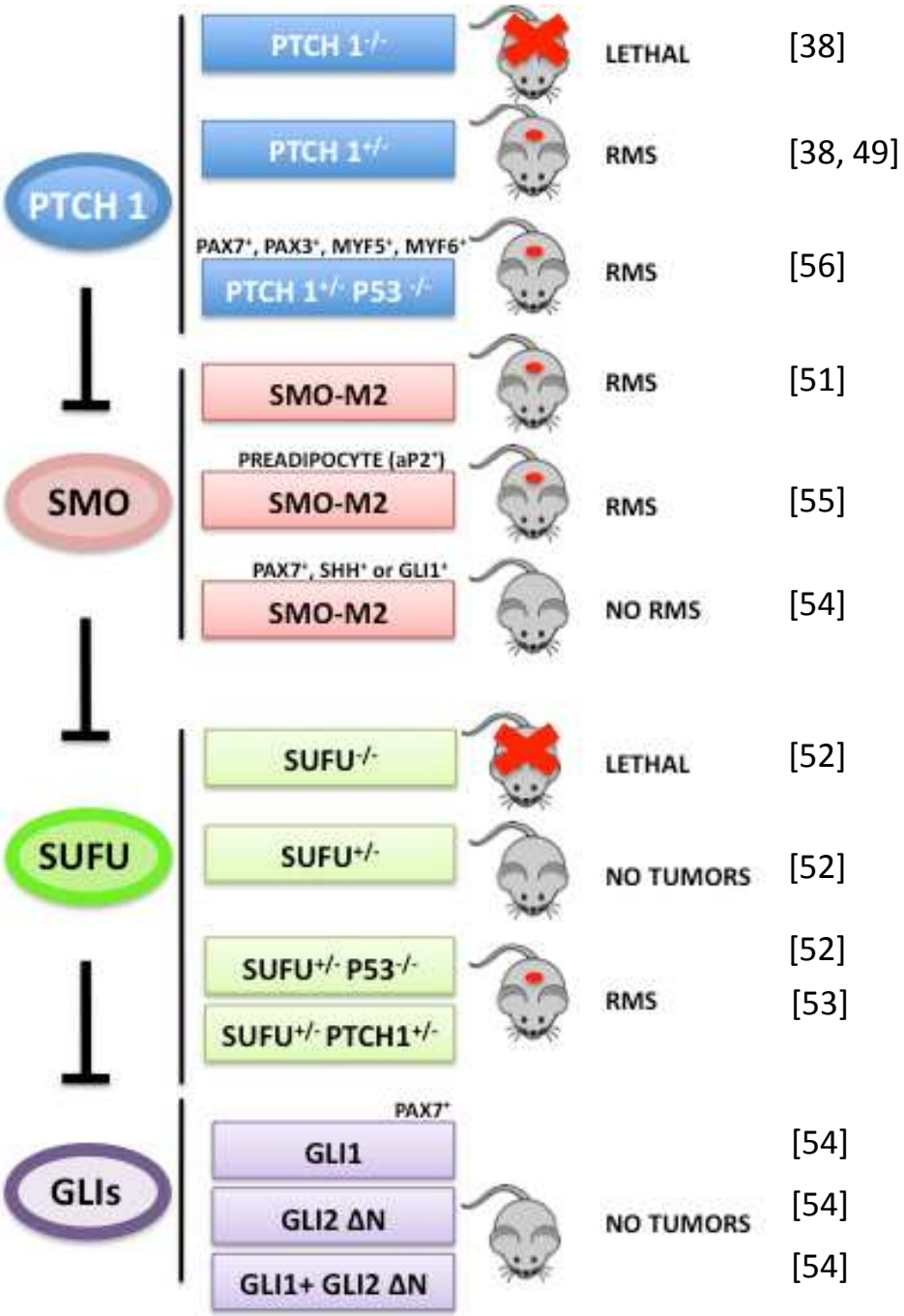
Figure 1.

Mouse models of Hh-driven RMS. Genetic manipulation of different components of Hh pathway such as Ptch1, Sufu and Smo leads to RMS tumorigenesis. Sufu inactivation favors RMS formation only in combination with P53 knock-out whereas $Ptch1^{+/-}$ can do the same without P53 ablation. Also combination of $Ptch1^{+/-}$ and $P53^{-/-}$ specifically in the myogenic compartment generates RMS. Similarly, constitutive and ubiquitous activation of Smo-M2 or specific expression in preadipocytes causes RMS but not if expressed in Hh epressing or responsive cells as well as in satellite cells. Finally, Gli1/2 activity in $Pax7^{+}$ cells is not sufficient for RMS initiation.

Figure 2.

Targeting Hh pathway. A wide range of Hh-directed antagonists can be used to inhibit Hh pathway including neutralizing antibodies against the ligands, SMO inhibitors and anti-GLI small molecules. Additionally, direct or indirect modulators of GLI1 activity are shown as potential approaches to specifically target this transcription factor.

Figure 1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PTCH 1 -/-
LETHAL
[38]
PTCH 1 +/-
RMS
[38, 49]
PAX7+, PAX3+, MYF5+, MYF6+
PTCH 1 +/- P53 -/-
RMS
[56]
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SMO-M2
RMS
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SMO-M2
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SMO-M2
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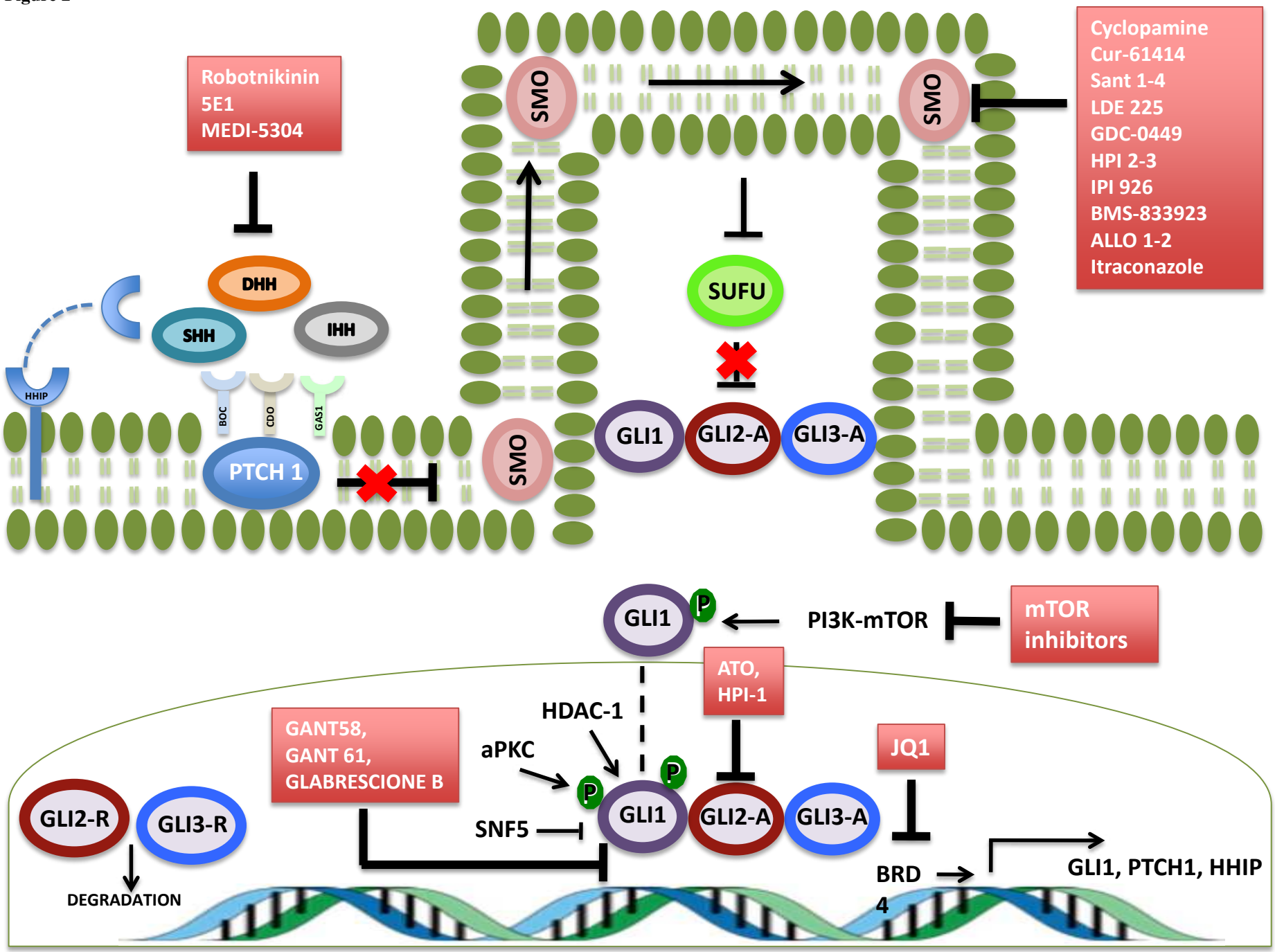
Figure 2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SMO
Cyclopamine
Cur-61414
Sant 1-4
LDE 225
GDC-0449
HPI 2-3
IPI 926
BMS-833923
ALLO 1-2
Itraconazole
SUFU
GLI1
GLI2-A
GLI3-A
GLI1
P
PI3K-mTOR
mTOR
inhibitors
ATO,
HPI-1
HDAC-1
aPKC
GANT58,
GANT 61,
GLABRESCIONE B
JQ1
GLI2-R
GLI3-R
SNF5
GLI1
GLI2-A
GLI3-A
DEGRADATION
BRD
4
GLI1, PTCH1, HHIP